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## Distribution of Octenyl Succinate Groups in Octenyl Succinic Anhydride Modified Waxy Maize Starch

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The location of octenyl succinate groups within octenyl succinic anhydride (OSA)-modified waxy maize (WM) starch granules was studied in order to better understand the relationship between the structure and physical properties of OSA starches. OSA starches of D.S. 0.03–0.11 were prepared by reaction between starch, OSA and NaOH in aqueous suspension; the native granular structure of starch was retained after reaction. Backscattered electron imaging of osmium-stained, sectioned OSA starch granules showed a uniform distribution of OSA groups over the cross-section of the granules. Anion-exchange chromatography of OSA starches solubilized in water showed that most of the amylopectin molecules contain some negative charge, suggesting that most of the starch granule is accessible to and reacts with OSA. However, after partial debranching with pullulanase, more of the resulting chains were neutral than would be expected on statistical grounds, suggesting heterogeneity in OSA substitution at the branch level. X-ray photoelectron spectroscopy suggested that the concentration of OSA groups on the immediate surface of the OSA starch granules was about 3–4 times that of the bulk.

**Keywords:** Starch, Starch ester, Octenyl succinate, Structure

### 1 Introduction

Starch alkenyl succinate esters are usually prepared commercially by the base catalyzed reaction of alkenyl succinic anhydrides with granular starch in aqueous suspension [1–3]. The chemical structure of starch octenyl succinate (OSA starch) is shown in Fig. 1 (substitution can occur at 2, 3, 6 positions). The latter is approved for food use at an OSA addition level of <3% (D.S. ~0.02).

With the incorporation of hydrophobic alkenyl groups into a normally hydrophilic starch molecule, the modified starch obtains surface active properties which are useful in stabilizing oil/water emulsions [1]. Unlike typical surfactants, starch alkenyl succinates form strong films at the

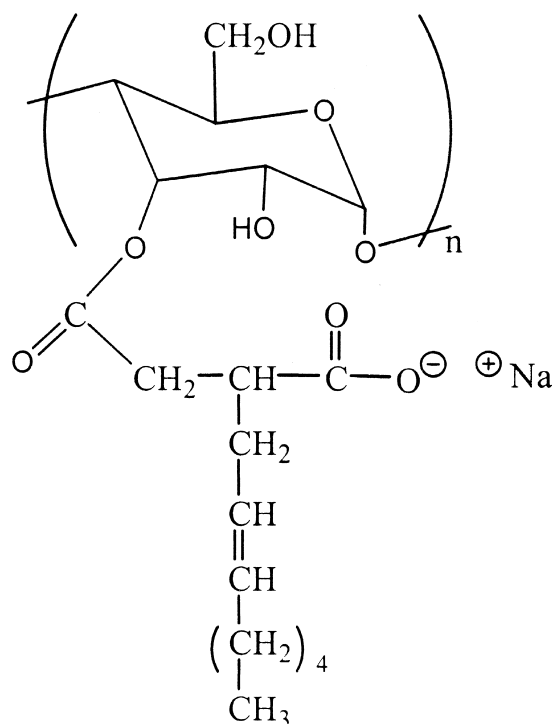


Fig. 1. Chemical structure of starch octenyl succinate.

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oil/water interface, giving the emulsion resistance to reagglomeration. As a result, aqueous solutions of starch alkenyl succinates and OSA starch, in particular, have been used to stabilize flavor concentrates in beverages, oil in salad dressings, and to encapsulate flavors, fragrances and vitamins in spray dried formulations [1, 4]. When treated with polyvalent metal ions, granular starch alkenyl succinates become quite water resistant and gain the property of free flow [1]. They are useful in a number of applications such as processing aids, body powders and lotions.

Some recent work has examined the effects of reaction conditions on the D.S. and efficiency of starch/alkenyl succinic anhydride reactions [5]. There has been, however, no published work on determining the location of OSA groups within the starch granule and within the amylopectin and amylose molecules themselves. Nor have there been studies on how changing reaction conditions or starch pretreatments might change the location of OSA groups. Such knowledge would be helpful in understanding the physical behavior of OSA starches as well as to lead to the design of modified starches with new and improved properties.

In this study, the distribution of OSA groups in sectioned OSA-waxy maize starch granules has been characterized, using osmium-staining and backscattered electron imaging. Near surface concentrations of OSA were determined by X-ray photoelectron spectroscopy. Finally, the heterogeneity of OSA substitution among different amylopectin molecules in solution was estimated using ion-exchange chromatography.

## 2 Experimental

### 2.1 Materials

Waxy maize starch (Amioca) and octenylsuccinic anhydride were gifts from National Starch and Chemical, Bridgewater, NJ. Diethylaminoethyl cellulose (DEAE), Sephacryl S-200HR and *K. Pneumoniae* pullulanase (2.2 U/mg solid, 0.15 mg protein/mg solid; remainder was buffer salts and stabilizer) were from Sigma Chemical. Osmium tetroxide (4% solution in water) and LR white resin (acrylic, medium hardness) and accelerator were from Polysciences, Warrington, PA.

## 3 Methods

### 3.1 Preparation of octenyl succinate esters of waxy maize starch

The preparation method has been described elsewhere [5] and is similar to that of Caldwell and Wurzburg [6]. Briefly, granular waxy maize starch was slurried with water at room temperature and pH was adjusted and maintained at 8.5–9.0 by addition of 2% NaOH solution.

Octenyl succinic anhydride was slowly added over about 6 h with stirring and pH adjustment. After reaction, the slurry was brought to pH 6.5 with 5% HCl, filtered, washed with water and air dried. Degree of substitution (D.S.) was determined by a titration method [5].

### 3.2 X-ray diffraction

X-ray powder diffraction analysis was performed with a Philips 1820 diffractometer operated at 40 kV, 30 mA with graphite filtered  $\text{CuK}_\alpha$  radiation and a theta compensating slit. Data were acquired in 0.05 degree two theta, 4 s steps. Samples were equilibrated at 23 °C and 50% relative humidity for 1 d, then lightly ground in an agate mortar prior to X-ray analysis.

### 3.3 Light microscopy

Unmodified waxy maize starch and 0.11 D.S. OSA-WM starch samples were placed in 0.05 M sodium phosphate buffer (pH 7.2) for 1 h, then held overnight at room temperature in 1% (w/v) solution of  $\text{OsO}_4$  in the same buffer. After rinsing three times with water, starch granules were photographed in water with a Zeiss Axioskop microscope using bright field optics.

### 3.4 Electron microscopy

Starch samples (~3 mg) were first placed in the bottom of a BEEM (polyethylene embedding) capsule and LR white resin (10 mL resin + 1 drop accelerator) was then added. Polymerization of the resin was carried out in an oven at 65 °C for 3 d. The resin-starch pieces were microtomed to give a flat surface using an American Optical 820 microtome and steel knife. Samples were stained with  $\text{OsO}_4$  vapor by placing the samples inside a sealed dish containing a beaker of  $\text{OsO}_4$  solution for 4 h. Samples were coated with carbon using a Mark II carbon coater (Ernest F. Fullam, Latham, NY) in order to minimize charging of the sample in the electron microscope. Backscattered electron (BSE) imaging was carried out with a JEOL JSM 6400V scanning electron microscope in the composition (backscattering) mode. Accelerating voltage was 10 kV and sample-detector distance was 9–11 mm.

### 3.5 Anion-exchange chromatography

Starch samples (13 mg) were dissolved in 5 mL mM potassium phosphate buffer, pH 6.0 by heating and magnetic stirring in a sealed reacti-therm vial (Pierce Scientific) for 20 min at 135 °C. Samples were then centrifuged at 9000  $\text{min}^{-1}$  for 10 min to sediment undissolved or precipitated materials. Approximately 4 mL of the supernatant was applied to a 3 × 1.5 cm column of DEAE cellulose equipped with a flow adaptor and peristaltic pump. The column was eluted with 20 mL of 1 mM phosphate

buffer and then 60 mL of a linear gradient of 1 mM phosphate buffer to 2 M NaCl + 1 mM phosphate buffer. Flow rate was 0.3 mL/min and fraction size was 3.4 mL. Fractions were assayed for total carbohydrate using the phenol-sulfuric acid assay [7].

Pullulanase treated starch samples were prepared by dissolving the starch (60 mg in 5 mL distilled water) as described above. Pullulanase (9 mg) was then added and pH was adjusted to 5.0 with 1 M NaOH. Samples were slowly magnetically stirred for 6 h at 23 °C then a second aliquot of 9 mg enzyme was added and stirring was continued for an additional 18 h. DEAE cellulose chromatography was performed as above.

### 3.6 Size exclusion chromatography

Size exclusion chromatography (SEC) was performed using a 100 × 1.5 cm column of Sephacryl S-200HR eluted with 0.1 M NaCl, 1 mM potassium phosphate buffer, 0.02% NaN<sub>3</sub>, pH 6.0. 1 mL of OSA-WM solution treated with pullulanase as described above was applied to the column. Fraction size was 2.4 mL and flow rate was 0.24 mL/min. Fractions were monitored for carbohydrate with the phenol-sulfuric acid method.

### 3.7 X-ray photoelectron spectroscopy

X-ray photoelectron spectra were collected with a PHI model 5400 X-ray photoelectron spectrometer (Physical Electronics, Inc., Eden Prairie, MN) equipped with small area electron extraction optics, a spherical capacitor electrostatic energy analyzer and a dual channel plate position sensitive detector. Starch powder samples were mounted with double stick Cu tape onto the sample holder. Samples were excited using characteristic MgK<sub>α</sub> X-rays, 1253.6 eV (15 kV, 400 W). Spectra were collected from a 1 mm<sup>2</sup> area of the surface at a 45° emission angle (relative to surface). Survey spectra were collected using a pass energy of 178.85 eV, 1 eV/step and multiplex spectra were collected using a pass energy of 35.75 eV, 0.1 eV/step. The binding energy scale was calibrated using Au4f<sub>7/2</sub> = 84.0 eV; the scale was shifted by ~2 eV to make C (CH<sub>2</sub>) = 285.0 eV to correct for charging effects.

Chemical state assignments were made based on tabulated energies [8]. Quantitative analyses were carried out by dividing elemental peak areas by the appropriate relative sensitivity factors as reported by the instrument manufacturer.

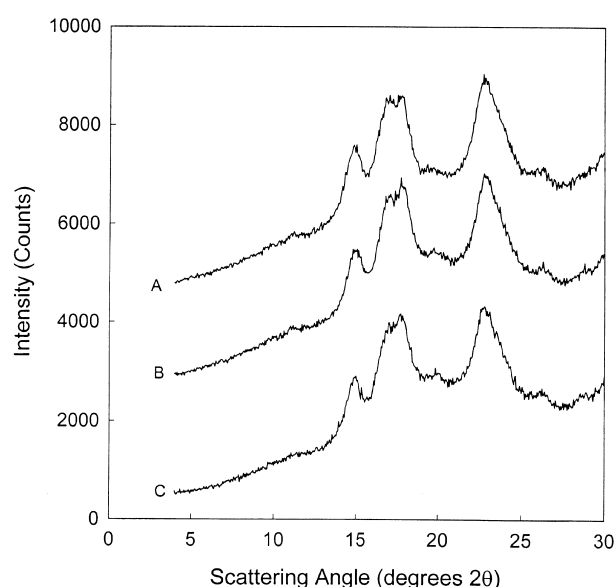
## 4 Results

X-ray powder diffraction scans of OSA-WM starch, D.S. 0.11 and 0.08 as well as unmodified waxy maize starch are shown in Fig. 2. All spectra show the native A-type crystalline pattern, indicating that reaction of waxy starch-

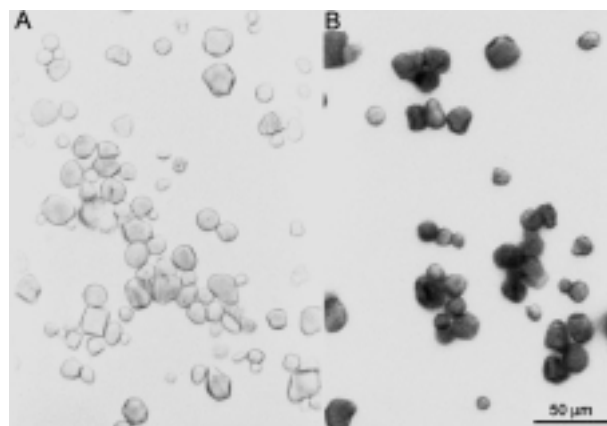
es with OSA in aqueous suspension causes no change in the crystalline structure of starch up to D.S. 0.11.

Light micrographs of Os-stained native waxy maize starch and OSA-WM D.S. 0.11 starch granules are shown in Fig. 3. The modified starch granules have a fairly uniform dark color while the unmodified starch granules are white in color. This indicates that Os has specifically stained the OSA groups and that these groups appear to be distributed throughout the OSA-WM starch granules. In order to obtain higher resolution images, electron microscopy of Os-stained sectioned granules was carried out.

Backscattered electron images of Os-stained cross-sections of OSA-WM starch granules having a D.S. of 0.11,



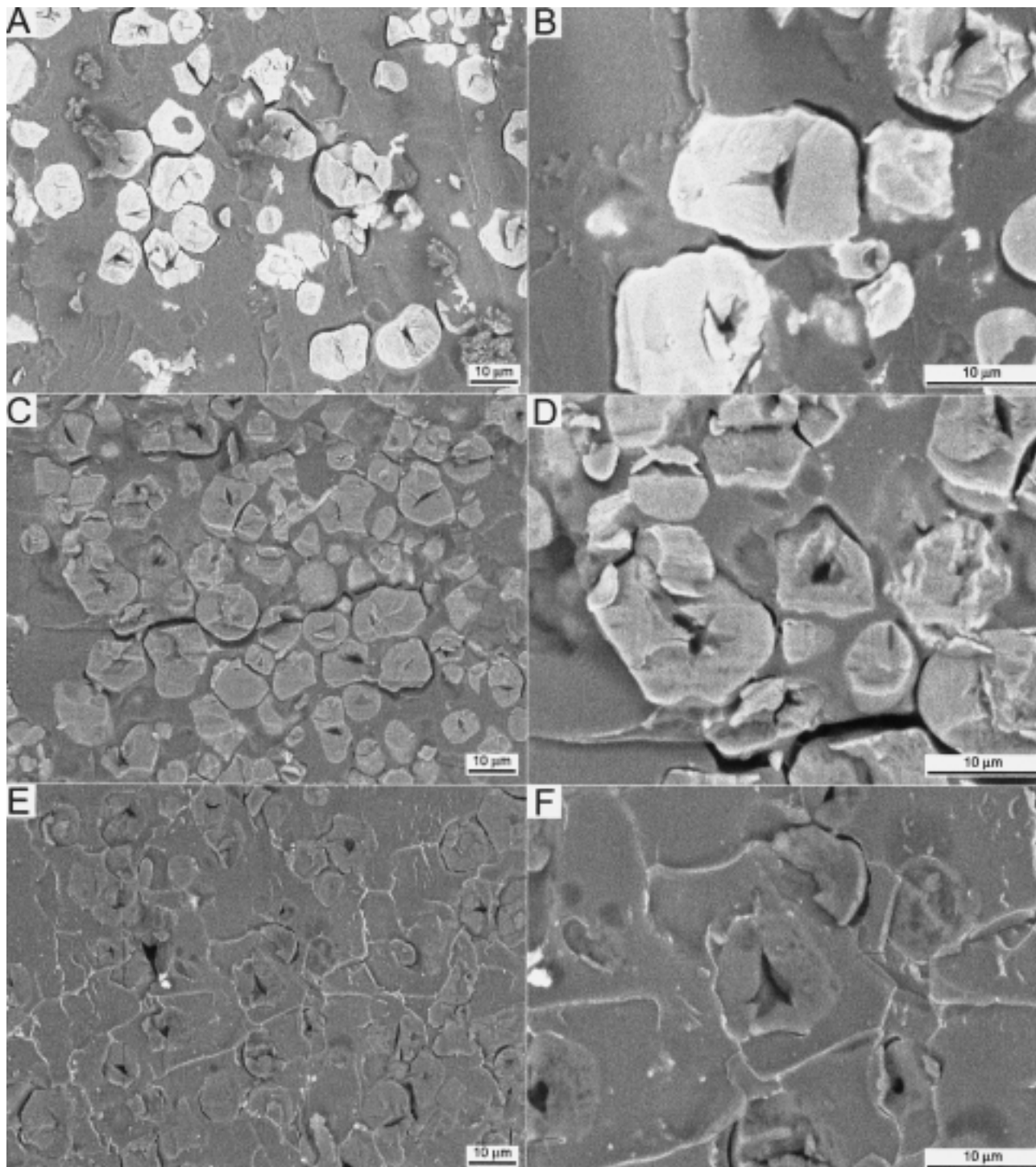
**Fig. 2.** X-ray powder diffraction scans of waxy maize starch (A), OSA-WM starch, D.S. 0.08 (B) and OSA-WM starch granules, D.S. 0.11 (C).



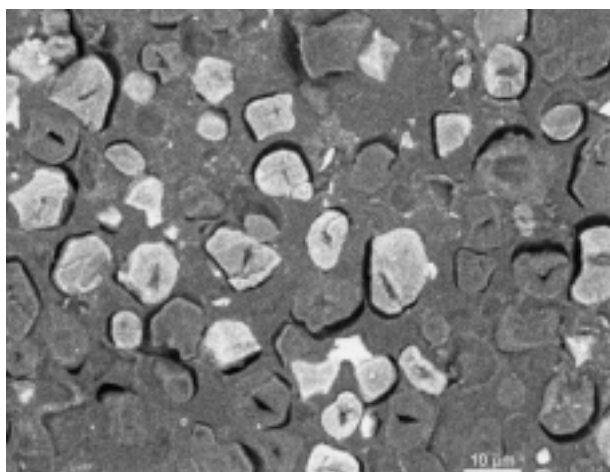
**Fig. 3.** Light micrographs of osmium-stained waxy maize starch (A) and OSA-WM starch, DS 0.11 (B).

0.03 and 0 (unmodified WM) are shown in Fig. 4. Light areas in the figures represent areas where  $\text{OsO}_4$  has oxidized the double bonds of OSA, resulting in the deposition of solid  $\text{OsO}_2$ . Since Os has a much higher electron density than C or O, incident electrons scatter strongly

from Os and thus its location can be ascertained. Both the D.S. 0.11 and 0.03 D.S. OSA-WM starches have rather uniform brightness or Os staining, suggesting that OSA groups are located throughout the starch granule. Staining is more intense for the D.S. 0.11 OSA-WM (A, B) than



**Fig. 4.** Backscattered electron images of osmium-stained OSA-WM starch granules, D.S. 0.11 (A, B), OSA-WM starch granules, D.S. 0.03 (C, D) and WM starch granules (E, F).

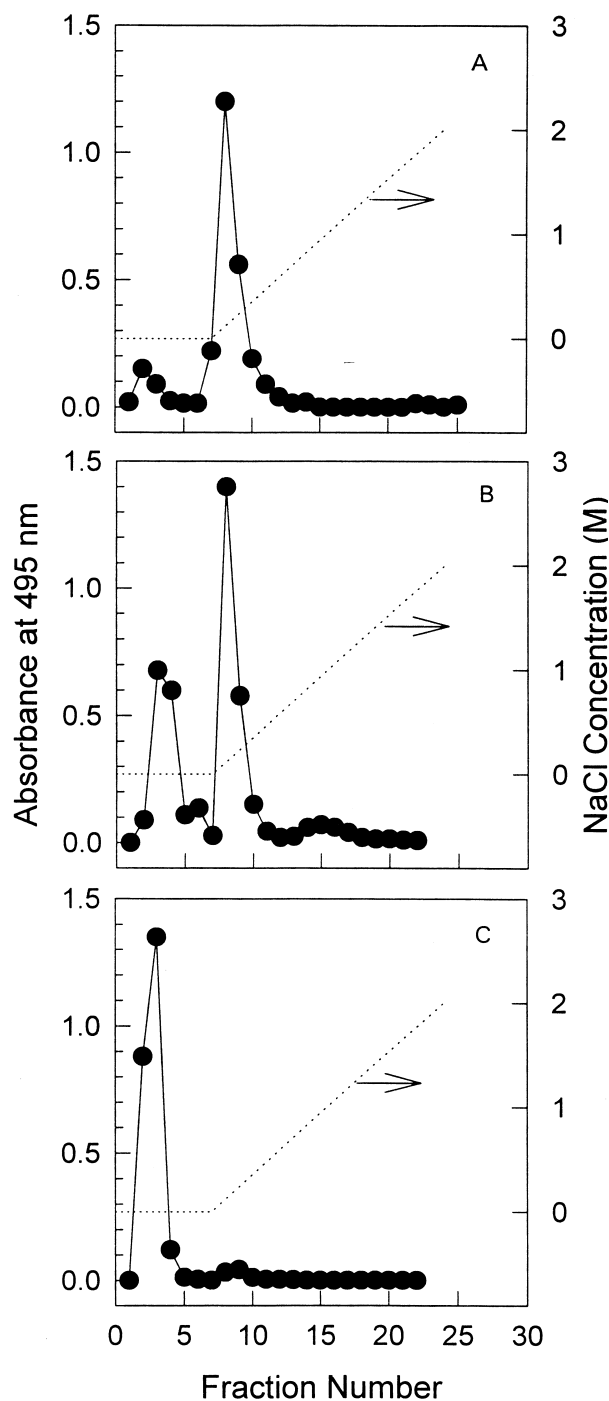


**Fig. 5.** Backscattered electron image of osmium-stained mixture of OSA-WM starch granules, D.S. 0.11 and unmodified WM starch.

for the D.S. 0.03 sample (C, D), as would be expected. Waxy maize starch having no OSA groups (E, F) showed no contrast with the background resin, confirming that the staining was due to OSA. As a check on this, a mixture of D.S. 0.11 OSA-WM and unmodified WM starch was embedded, sectioned, stained with  $\text{OsO}_4$  and examined in the electron microscope. Stained and unstained granules could be clearly distinguished (Fig. 5).

To characterize the distribution of OSA groups on the molecular level, OSA-WM molecules were separated on the basis of charge using DEAE cellulose chromatography (Fig. 6A–C). These chromatograms represent only about 25% of the original material, however, because the rest was removed by centrifugation prior to chromatography. Solutions were relatively clear after heating at 135 °C but became cloudy after cooling. This suggests that most of the OSA-WM samples were solubilized on heating but aggregated and precipitated as hydrophobic associations formed between starch molecules on cooling. Thermally reversible gelation of hydrophobically modified starches has been described previously [9]. The part of the samples which was insoluble and removed by centrifugation may have had a relatively higher molecular weight or more OSA groups than the solubilized portion. In contrast, approximately 72% of unmodified waxy starch was present in the supernatant after centrifugation.

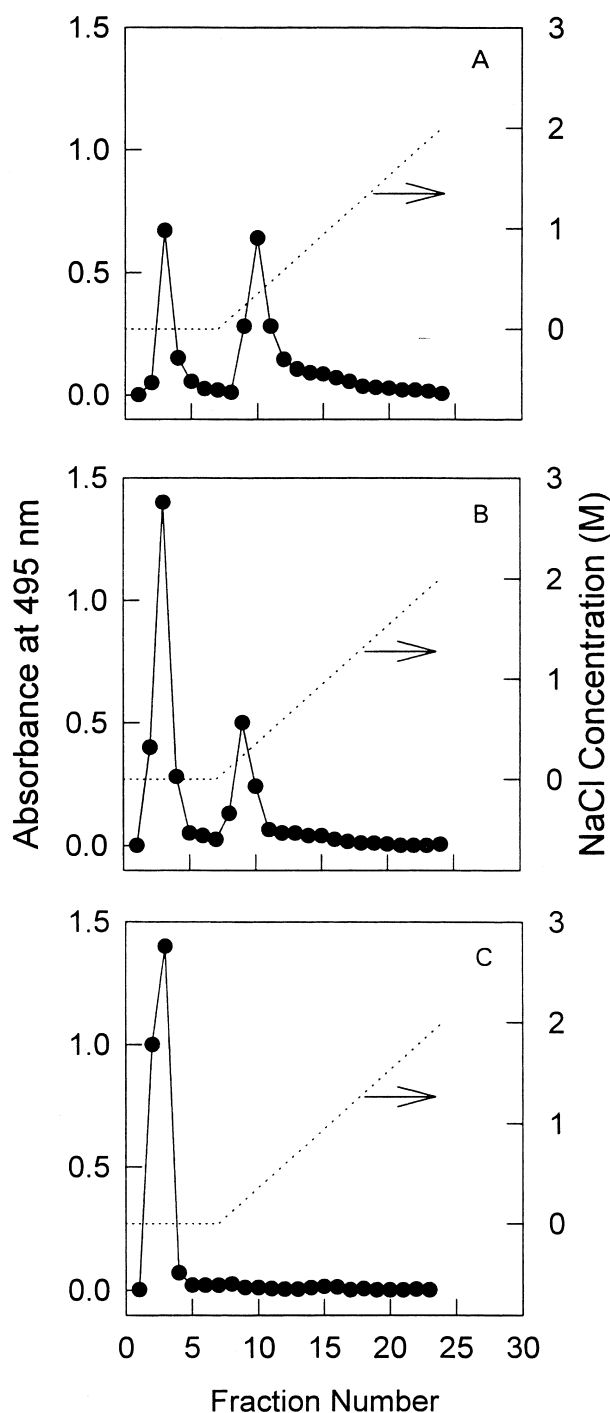
Neutral, unreacted starch molecules (Fig. 6C) were eluted off the column first with 1 mM buffer while those having a negative charge from OSA were eluted afterwards with a 0–2 M NaCl gradient. Most (~90%) of the starch in the 0.11 D.S. OSA-WM sample had some negative charge while about 2/3 of the starch in the 0.03 D.S. sample was charged. Moreover, the peak eluted by the gradient was



**Fig. 6.** DEAE cellulose column chromatograms of OSA-WM starch, D.S. 0.11 (A), OSA-WM starch, D.S. 0.03 (B), and WM starch (C).

sharp, suggesting little charge heterogeneity. Although some caution is warranted due to the large amount of sample which was removed during centrifugation, the data suggest that most of the molecules in OSA-WM starch have some covalently attached OSA groups in agreement with the microscopy results.

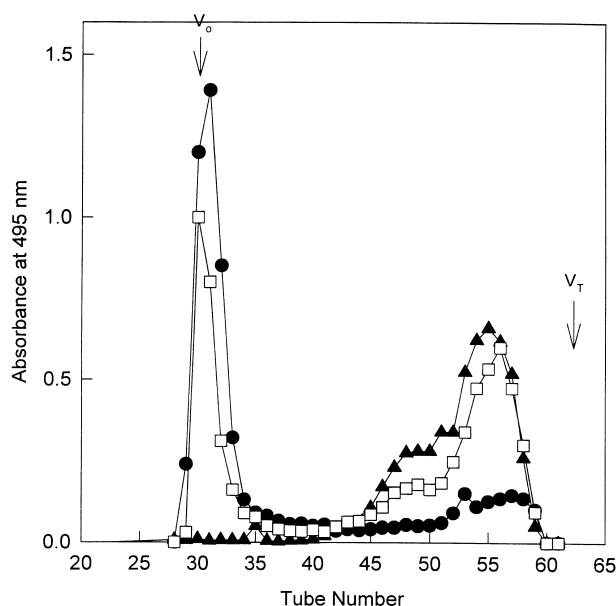
DEAE cellulose chromatograms of pullulanase digested OSA-WM are shown in Fig. 7. No material was lost on centrifugation prior to chromatography so the results in Fig. 7 represent the whole sample. Since the pullulanase preparation used contained a small amount of carbohydrate stabilizer, it was also run on the DEAE cellulose col-



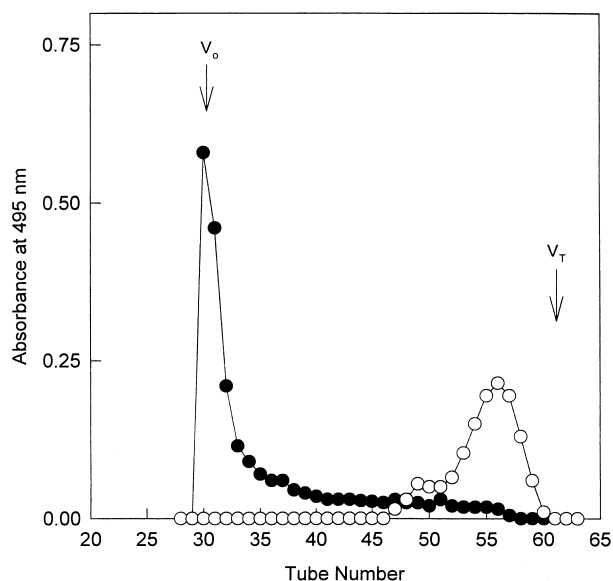
**Fig. 7.** DEAE cellulose column chromatograms of pullulanase treated OSA-WM starch, D.S. 0.11 (A), OSA-WM starch, D.S. 0.03 (B), and WM starch (C).

umn and the small peak at fractions 2–4 was subtracted from the curves shown in Fig. 7. Neutral starch branches or molecular fragments make up 30% of the D.S. 0.11 sample and 65% of the D.S. 0.03 sample. The digestion of  $\alpha$ -1,6 linkages of OSA-WM starch was probably not complete, however, because solutions were still slightly viscous (see below). Thus, the proportion of neutral versus charged branches shown in Fig. 7 is likely an underestimate.

To estimate the extent of debranching in pullulanase digested OSA-WM, size exclusion chromatography (SEC) using Sephacryl S-200 was performed. A peak corresponding to the carbohydrate stabilizer eluting around the total volume of the column was subtracted from the results. As shown in Fig. 8, unmodified WM is completely digested by pullulanase into starch branches which elute as a shoulder at about fraction 48 and main peak at fraction 55. For OSA-WM of D.S. 0.03, about 37% of the sample elutes near the void volume and thus these starch molecules have a large hydrodynamic volume and hence high molecular weight. For the D.S. 0.11 sample, about 72% of the sample elutes near the void volume. SEC chromatograms of the neutral and charged components of pullulanase digested OSA-WM, D.S. 0.11 (DEAE cellulose fraction #'s 3 and 10, respectively, from Fig. 7A) are shown in Fig. 9. It is apparent that the charged, OSA modified portion of the sample consists of large molecules while the neutral portion consists of individual branches. Thus, pullulanase selectively removes unmodified branches from OSA-WM, leaving relatively large mole-



**Fig. 8.** Sephacryl S-200HR chromatography of pullulanase treated OSA-WM starch, D.S. 0.11 (●), OSA-WM starch, D.S. 0.03 (□), and WM starch (▲).



**Fig. 9.** Sephacryl S-200HR chromatography of DEAE fractions 3 (○) and 7 (●) of pullulanase treated OSA-WM starch, D.S. 0.11 (see Fig. 7A).

cules which contain the OSA groups. The average D.S. of the latter are calculated to be 0.08 for the D.S. 0.03 sample and 0.15 for the D.S. 0.11 sample.

Based on random chance, the probability that a starch branch of length  $n$  in a collection of  $x$  starch branches will not be derivatized by an OSA molecule is:

$$p = (1 - 1/x)^{n \times \text{D.S.}} \quad (1)$$

Thus for D.S. = 0.11, an average branch length  $n = 23$  [10] and  $x = 1000$  (an arbitrary large number), one calculates  $p = 0.08$ . Therefore, 92% of the branches in a D.S. 0.11 OSA WM starch molecule would be expected to have at least one OSA group if all branches had equal reactivity. Since it was experimentally determined above that 72% or less of the branches or fragments contain an OSA group, it appears that some branches are more accessible or reactive towards OSA than others. There is a long tail extending to higher salt concentrations in Fig. 7A and 7B, also suggesting that there are some fragments with a higher charge density than others.

Surface compositions of OSA-WM starches as determined by X-ray photoelectron spectroscopy (XPS) are given in Tab. 1. Due to the intense absorption of emitted electrons by matter, the XPS technique is sensitive to only the outer 30–50 Å thick surface shell [11]. In order to determine the molecular composition from data in Tab. 1, it can be assumed that the elements observed arise from the glucose units of starch, OSA, etc. A series of simultaneous equations can be written as follows:

**Tab. 1.** Surface elemental composition of OSA-waxy maize starches (number fraction  $\times 100$ ).

D.S.	C	O	Na	N	P	Si
0.11	65.6	31.9	1.8	0.67	0	0
0.03	62.5	35.2	0.69	0.92	0	0.74
0	63.5	35.1	0.0	0.72	0.04	0.68

$$2s + h + 6g + 4.8p + 12a = n_C \quad (2)$$

$$s + 5g + 1.9p + 3a = n_O \quad (3)$$

$$a = n_{Na} \quad (4)$$

$$1.3p = n_N \quad (5)$$

$$s = n_{Si} \quad (6)$$

where  $n$  represents the number fraction  $\times 100$  of an experimentally observed element,  $g$  is the number of glucose residues,  $a$  is the number of OSA groups,  $p$  is the number of amino acid residues,  $s$  is the number of silicon oil molecules ( $C_2H_6SiO$ ) and  $h$  is the number of hydrocarbon ( $CH_2$ ) groups. Hydrocarbons and silicon oils are common contaminants found on the surfaces of many materials exposed to ambient air [12]. Proteins are found in small amounts on starch granules and were approximated by the formula,  $C_{4.8}O_{1.9}N_{1.3}H_{7.7}$  [12].

The solutions to equations 2–6 for OSA-WM and unmodified WM starch are given in Tab. 2. Taking the ratio of  $a/g$ , values for the surface D.S. of 0.35 and 0.11 were calculated for the (bulk) D.S. 0.11 and 0.03 samples, respectively. This suggests that the surface is enriched with OSA groups by a factor of 3–4 over that of the bulk granule.

**Tab. 2.** Calculated molecular composition of surfaces of OSA-waxy maize starches (relative number of molecules).

D.S.	$g$	$a$	$h$	$p$	$s$
0.11	5.1	1.8	11	0.5	0.0
0.03	6.2	0.7	12	0.7	0.7
0	6.7	0	19	0.6	0.7

$g$  = glucose residue ( $C_6O_5H_{10}$ );  $a$  = OSA ( $C_{12}O_3H_{17}Na$ );  $h$  = hydrocarbon ( $CH_2$ );  $p$  = protein ( $C_{4.8}O_{1.9}N_{1.3}H_{7.7}$ );  $s$  = silicon oil ( $C_2H_6SiO$ ).

## 5 Discussion

Huber and BeMiller [13, 14] have recently reported on the architecture and permeability of corn starch granules. They have clearly shown that starch granules have many narrow channels leading from the surface to a central cavity. The latter appears to be rather amorphous in structure since it will swell nearly shut in water but expand in dehydrating solvents such as methanol. The central area

around the hilum is also known to be the first part of the granule to undergo gelatinization and degradation by enzymes and acid. The channels and cavity are accessible to particles at least as large as 50 nm, as demonstrated by the colloidal gold experiments. They also showed that, using backscattered imaging, merbromin (molecular weight 700) in aqueous solution penetrated evenly throughout starch granules over a period of 1.5 h. Experiments over a shorter time showed that merbromin first permeated the central cavity then diffused into the rest of the granule from inside out.

The reaction rate of OSA with starch in aqueous suspension at pH 8 is slow, normally requiring 6–8 h. The rate of diffusion of OSA into the granule would be expected to occur much faster than this based on the experiments described above. Thus, the relatively even distribution of OSA in the granule probably occurs by the diffusion of OSA into the starch granule followed by reaction with starch. *Huber and BeMiller* [15] have also found that reagents that react slowly with starch such as propylene oxide give a mostly uniform distribution while those which react rapidly such as  $\text{POCl}_3$  give more surface reaction. Using XPS, *Hamunen* [16] also found that surface concentrations of N from reaction of potato starch with 1,2-epoxypropyltrimethylammonium chloride were similar to or less than interior N concentrations.

However, the solubility of OSA in water is low so that there is probably a mixture of OSA droplets and OSA dissolved in water. The droplets could react with the granular surface or, depending on their size, travel into the channels and interior cavity. Presumably only OSA which is molecularly dissolved or extremely finely dispersed could penetrate into the bulk of the granule. It would seem likely that the granular surface of OSA-WM has a higher D.S. than the bulk because the granular surface can react with OSA droplets as well as with dissolved OSA. It has recently been claimed that higher D.S. starch alkenyl succinates can be made more rapidly by better dispersing the OSA in water prior to reaction [17].

Starch alkenyl succinates have higher viscosities in aqueous suspension, lower gelatinization temperatures and less tendency to retrograde than unmodified starch [1]. This behavior would be consistent with our finding that a high proportion of starch molecules have some chemically bound OSA. The hydrophobic nature of OSA starch granules is also consistent with our finding of a relatively high surface derivatization of starch with OSA.

Previous work on modified starches have shown a preference of substituents for the branched regions of amylopectin. For a hydroxypropyl (HP) amylopectin of D.S. 0.1, about 40% of the debranched amylopectin contained

no HP group [18]. These were, however, completely hydrolyzed by  $\beta$ -amylase, indicating that they were linear chains. It is likely that chemical reagents cannot penetrate the crystalline domains of starch which are thought to be made up of short (D.P. 10–15) branches. Similar results were obtained for methylated potato amylopectin [19]. Thus, our finding that about 1/4 of the amylopectin branches in the D.S. 0.11 sample have no OSA group is consistent with the hypothesis that they represent the crystalline outer branches of amylopectin. The observation that X-ray powder diffraction scans of the OSA starches show a A-type pattern which is very similar to the unmodified starch also supports the view that the crystalline domains of starch are not substantially altered by derivatization with OSA.

## 6 Conclusions

This study has suggested that OSA groups are probably distributed in the interior, amorphous domains of amylopectin molecules as well as on the outside of the granule. This leads to the question of how altering the distribution might affect physical properties such as emulsification activity. Modification of the outside branches might improve activity by making the OSA groups more accessible rather than being buried inside. Also, would an OSA amylose or a starch having substitution restricted to one hydroxyl position have altered properties? What new physical properties might be obtained with a high D.S. OSA starch? These questions await further research.

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